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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ANDERSEN, Morten, Birket [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsvaerd (DK). YDE, Birgitte [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsvaerd (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).

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(54) Title: LACCASE/PEROXIDASE TREATMENT OF LIGNOSULPHONATES

(57) Abstract

A method of producing a gel, comprising adding a phenol oxidizing enzyme system to a solution of lignosulphonates (optionally substituted), and holding for a sufficient time to form a gel.

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LACCASE/PEROXIDASE TREATMENT OF LIGNOSULPHONATES

FIELD OF INVENTION

The present invention relates to a method for the production of a gel of lignosulphonates and/or derived lignosulphonates.

BACKGROUND OF THE INVENTION

Lignosulphonates (also called lignin sulphonates or sulphonated lignins) are lignins from the sulfite process. Lignin is a waste product in relation to paper production, so many attempts have been made trying to utilize it, either as such or in a modified form. Thus, as an example, lignin has been modified enzymatically in order to function as a glue in particle boards, see for instance A. Hüttermann et al. in Lignin: Properties and Materials ACS Symp. Ser. 397 (1989), Chapter 27, pp. 361-370. S. Sarkanen and W. G. Glasser (Eds). Washington D.C: American Chemical Society.

SUMMARY OF THE INVENTION

In this invention it is surprisingly found that a gel comprising lignosulphonates and/or derived lignosulphonates may be produced by

a) adding a phenol oxidizing enzyme system to a solution of lignosulphonates (optionally substituted), and

b) holding for a sufficient time to form a gel.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows a theoretical curve from a Texture Profile Analysis showing hardness, adhesiveness and cohesiveness.

Fig. 2 shows a curve from a Texture Profile Analysis of a gel produced as described in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

Gel Formation

According to the invention a gel material of lignosulphonates may be formed by addition of a phenol oxidizing enzyme system.

It is believed that the overall gel formation proceeds through polymerization of lignosulphonates, presumably by formation of phenoxy radicals of hydroxylated aromatic constituents. Subsequent and increasing crosslinking of phenoxy radicals eventually gives a three-dimensional structure of lignosulphonates, and this forms a gel. According to the invention it may take up to 5-10 days to form this three-dimensional structure of lignosulphonates depending on, inter alia, temperature, the phenol oxidizing enzyme used, and the concentration of the involved reactants, so by using, e.g., a higher enzyme concentration the reaction may proceed much faster. It is demonstrated in Example 2 that the gel can be formed within 1 hour.

Instead of using lignosulphonates, phenols may be used, or a mixture of lignosulphonates and phenols.

It is well known that the physical properties of a gel is widely different to those of a non-gelated solution. These differences may be characterized by a Texture Profile Analysis as described below.

According to the present invention the term "gel" is used when the hardness is $\geq$ 0.15 N, the hardness being defined by the Texture Profile Analysis as described herein.

Texture Profile Analysis

Gels formed from lignosulphonates by addition of a phenol oxidizing enzyme system, optionally an oxidizable substrate, may be characterized by a Texture Profile Analysis (TPA) as described by M. C. Bourne in Food Technology, 1978,

pp.62-66 and p.72. A TPA is a time dependent measurement of the deformation force (given in Newton, N) performed on the gel by a cylindrical probe with a given area and of uniform size.

According to the invention a probe area of 314 mm^2 is used and the TPA is performed on a gel, placed in a teflon form (6 x 6 cm), as two succeeding compressions, interrupted by elevating the plate to the surface of the gel. From the time dependent force profile, textural parameters, hardness, adhesiveness and cohesiveness, are extracted based on graphic definitions as shown in Fig. 1. Hardness is the height, given in N, of the first peak. Adhesiveness is the negative area A_3, given in Ns, from the first compression. Cohesiveness is the area ratio A_2/A_1 of the two compressions. The textural parameters serve to describe the gel.

Calibration: Before the measurement the apparatus is calibrated in order to define the surface and the bottom of the gel. The latter is measured by descending the plate into an empty teflon form until the bottom is reached. The surface of the gel is defined as the contact force needed to start the compression (trigger point). The contact force is set sufficiently low so that the force exerted by air, while descending the plate onto the gel, does not trigger the compression.

Measurements: The measurement is performed by descending the plate into the gel starting from the triggering point. The plate is pressed into the gel with a constant speed (mm/sec) until a given % deformation of the gel height has been reached. From this point the plate is raised back to the triggering point and the procedure is repeated. During these two compressions the deformation force is recorded, and the result of the compressions is given as a time dependent force profile.

Lignosulphonates

Wood consists mainly of cellulose, hemicellulose and lignin. In order to make the cellulose practically useful, e.g. for paper manufacture, the lignin must first be removed. This may be done by extracting the lignin in a sulfite cooking process, whereby the lignosulphonates are produced. The

lignosulphonates will differ depending on, inter alia, which species of wood are used, the exact cooking method and the subsequent purification method(s). The lignosulphonates may be produced as powders of calcium-, ammonium-, magnesium-, sodium- or chromium-based salts of lignosulphonates.

Phenol Oxidizing Enzyme System

The enzyme system used in the invention consists of a suitable peroxidase together with a hydrogen peroxide source or a suitable oxidase together with O_2. Suitable enzymes are those which oxidize aromatic compounds such as phenols and lignin.

Examples of suitable enzymes are peroxidases (EC 1.11.1.7), catechol oxidases (EC 1.10.3.1) and laccases (EC 1.10.3.2).

Preferably, the peroxidase employed in the method of the invention is producible by plants (e.g. horseradish or soybean peroxidase) or microorganisms such as fungi or bacteria. Some preferred fungi include strains belonging to the subdivision Deuteromycotina, class Hyphomycetes, e.g. Fusarium, Humicola, Tricoderma, Myrothecium, Verticillum, Arthromyces, Caldariomyces, Ulocladium, Embellisia, Cladosporium or Dreschlera, in particular Fusarium oxysporum (DSM 2672), Humicola insolens, Trichoderma resii, Myrothecium verrucana (IFO 6113), Verticillum alboatrum, Verticillum dahlie, Arthromyces ramosus (FERM P-7754), Caldariomyces fumago, Ulocladium chartarum, Embellisia alli or Dreschlera halodes.

Other preferred fungi include strains belonging to the subdivision Basidiomycotina, class Basidiomycetes, e.g. Coprinus, Phanerochaete, Coriolus or Trametes, in particular Coprinus cinereus f. microsporus (IFO 8371), Coprinus macrorhizus, Phanerochaete chrysosporium (e.g. NA-12) or Trametes (previously called Polyporus), e.g. T. versicolor (e.g. PR4 28-A).

Further preferred fungi include strains belonging to the subdivision Zygomycotina, class Mycoraceae, e.g. Rhizopus or Mucor, in particular Mucor hiemalis.

Some preferred bacteria include strains of the order Actinomycetales, e.g. Streptomyces spheroides (ATTC 23965), Streptomyces thermoviolaceus (IFO 12382) or Streptoverticillum verticillium ssp. verticillium.

Other preferred bacteria include Bacillus pumilus (ATCC 12905), Bacillus stearothermophilus, Rhodobacter sphaeroides, Rhodomonas palustri, Streptococcus lactis, Pseudomonas purrocinia (ATCC 15958) or Pseudomonas fluorescens (NRRL B-11).

Further preferred bacteria include strains belonging to Myxococcus, e.g. M. virescens.

The peroxidase may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said peroxidase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the peroxidase, in a culture medium under conditions permitting the expression of the peroxidase and recovering the peroxidase from the culture.

Particularly, a recombinantly produced peroxidase is a peroxidase derived from a Coprinus sp., in particular C. macrorhizus or C. cinereus according to WO 92/16634, or a variant thereof, e.g., a variant as described in WO 94/12621.

According to the invention microbial laccase (EC 1.10.3.2) is preferred. The microbial laccase may be derived from bacteria or fungi (including filamentous fungi and yeasts). The microbial laccase is preferably obtained from a fungus.

Some preferred fungi include strains belonging to the subdivision Basidiomycotina and to the subdivision Ascomycotina. Suitable examples include a laccase derivable from a strain of Aspergillus, Neurospora, e.g., N. crassa, Podospora, Botrytis, Collybia, Fomes, Lentinus, Pleurotus, Trametes, e.g., T. villosa and T. versicolor, Rhizoctonia, e.g., R. solani, Coprinus, e.g., C. plicatilis and C. cinereus, Psatyrella, Myceliophthora, e.g., M. thermophila, Schytalidium, e.g., S.thermophilum, Polyporus, e.g., P. pinsitus, Phlebia, e.g., P. radita (WO 92/01046), Coriolus, e.g., C.hirsutus (JP 2-238885),.

Hygrophoropsis, Agaricus, Vascellum, Crucibulum, Myrothecium, or Sporormiella.

In particular laccases derivable from T. villosa, T.versicolor or M. thermophila are preferred.

The laccase may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said laccase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the laccase in a culture medium under conditions permitting the expression of the laccase and recovering the laccase from the culture.

Before the enzyme is added to a solution of lignosulphonates, the pH of the solution may be adjusted to the optimum of the enzyme.

The amount of peroxidase should generally be in the range of from 5-1000 PODU per g of lignosulphonates, preferably in the range of from 50-500 PODU per g of lignosulphonates (PODU is the unit of peroxidase activity as defined below).

The amount of laccase should generally be in the range of from 0.1-100 LACU per g of lignosulphonates, preferably in the range of from 1-10 LACU per g of lignosulphonates (LACU is the unit of laccase activity as defined below).

The hydrogen peroxide source may be hydrogen peroxide or a precursor of hydrogen peroxide, preferably perborate or percarbonate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and its substrate, or a peroxycarboxylic acid or a salt thereof. Different peroxidases will often have different tolerances towards hydrogen peroxide, but it will normally be an advantage to keep the hydrogen peroxide concentration low; this may be done by adding the hydrogen peroxide continuously during the gelating period, but if the peroxidase enzyme does not loose its activity the hydrogen peroxide source may also be added to the solution of lignosulphonates at one time or, for instance, at a few times.

The hydrogen peroxide source may be added to the solution of lignosulphonates in a total amount corresponding to

0.05-5 mmoles of hydrogen peroxide per g of lignosulphonates.

If the phenol oxidizing enzyme system consists of an oxidase together with O_2, molecular oxygen from the atmosphere may be sufficient, typically by stirring the solution of lignosulphonates. If more O_2 is needed, additional oxygen may be added, typically by bubbling the solution of lignosulphonates with air or oxygen. Optionally, pH may be adjusted to the optimum of the oxidase.

Additionally an oxidizable substrate such as an organic compound, such as a phenolic compound, e.g. p-hydroxybenzene sulphonate, or one of the compounds disclosed in WO 94/12621, may be added in order to enhance the effect of the phenol oxidizing enzyme system. The amount of oxidizable substrate may correspond to a concentration in the solution of lignosulphonates of between 0.01 μM and 100 μM.

Determination of Peroxidase Activity (PODU)

Peroxidase activity is determined from the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by hydrogen peroxide. The greenish-blue colour produced is photometered at 418 nm. The analytical conditions are 0.88 mM hydrogen peroxide, 1.67 mM ABTS, 0.1 M phosphate buffer, pH 7.0, 30°C, 3 minutes reaction.

1 peroxidase unit (PODU) is the amount of enzyme that catalyses the conversion of 1 μmole hydrogen peroxide per minute at these conditions.

Determination of Laccase Activity (LACU)

Laccase activity is determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 μM syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 min. reaction time.

1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1 μmole syringaldazin per minute at these conditions.

Applications

The gel of the invention may be useful in any application where a material is needed which has the ability both to absorb and to emit water or organic solvents or mixtures of water and organic solvents. The gel may for example be useful in treatment of waste water by absorbing unwanted chemicals.

The present invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLE 1

Solution of Lignosulphonates

Stock solutions of lignosulphonates were made in water by gradual addition of the required amount of lignosulphonates to the desired volume of water giving a brown viscous solution.

The concentration of aqueous lignosulphonates is given as weight-%:

(Mass of lignosulphonates/
(Mass of lignosulphonates + Mass of water)) · 100%

The pH of a lignosulphonate solution varies from approx. pH 2 to pH 6 depending on concentration; pH is lower the more concentrated the lignosulphonate solution is.

Gel Formation with Laccase

A gel forming solution was produced by mixing 55 ml of lignosulphonate stock solution 46% (w/w) with 78 ml of water and 2.0 ml of laccase solution with an activity of 81 LACU/ml. (The used lignosulphonate was a calcium-based lignosulphonate, produced from spruce wood, Wafex P, available from Borregaard Lignotech, Norway. The used laccase was a Trametes villosa laccase, SP 504, available from Novo Nordisk A/S).

The concentration of each ingredient in the gel forming solution was then:

Lignosulphonate: 19% (w/w)
Laccase: 6.3 LACU per g lignosulphonate

The mixture was poured into a teflon form, which was isolated in a humid plastic chamber to avoid evaporation of water. The chamber was left at room temperature. A gel as defined in the present invention was obtained within 5-6 days. A texture profile analysis was performed as described above, and Fig. 2 gives the result of the analysis:

Conditions:
% Deformation: 40%
Rate: 2 mm/sec
Gel Height: 1.7 mm.
Contact force: 0.05 N
Probe: 314 mm^2

Results:
Hardness: 9.3 N
Adhesiveness: -0.081 Ns
Cohesiveness: 1.068

Example 2

Gel Formation with Peroxidase

Gel formation with peroxidase was tested by using the method described in Example 1, but instead of laccase/oxygen as the phenol oxidizing enzyme system peroxidase/hydrogen peroxide was used. The following concentrations in the gel forming solution were used:

Lignosulphonate: 21% (w/w)
Peroxidase: 450 PODU per g lignosulphonate
Hydrogen peroxide (in total):

0.4 mmoles per g lignosulphonate (added continuously through a period of 47 minutes at a rate of 8.5 μmoles per g lignosulphonate per minute).

The used lignosulphonate was Ultrazine CA, available from Borregaard Lignotech, Norway, and the used peroxidase was a Coprinus cinereus peroxidase, SP 502, available from Novo Nordisk A/S.

A gel as defined in the present invention was obtained within 47 minutes.

A texture profile analysis was performed as described above giving the following results:

Conditions:

% Deformation:	40%
Rate:	2 mm/sec
Gel Height:	1.7 mm.
Contact force:	0.05 N
Probe:	314 mm^2

Results:

Hardness:	0.2 N
Cohesiveness:	0.5

CLAIMS

1. A method of producing a gel, comprising:

a) adding a phenol oxidizing enzyme system to a solution of lignosulphonates (optionally substituted), and

b) holding for a sufficient time to form a gel.

2. A method according to claim 1, in which the phenol oxidizing enzyme system is an oxidase together with oxygen.

3. A method according to claim 2, in which the oxidase is an enzyme selected from the group consisting of laccase and catechol oxidase.

4. A method according to claim 2 or 3, wherein the laccase is derived from Trametes, e.g. Trametes villosa.

5. A method according to claim 4, wherein the amount of laccase is in the range from 0.1-100 LACU per g of lignosulphonates.

6. A method according to claim 1, in which the phenol oxidizing enzyme system is a peroxidase and a hydrogen peroxide source.

7. A method according to claim 6, wherein the peroxidase is horseradish peroxidase, soybean peroxidase or a peroxidase enzyme derived from Coprinus, e.g. C. cinereus, or from Bacillus, e.g. B. pumilus.

8. A method according to claim 6 or 7, wherein the amount of peroxidase is in the range from 5-1000 PODU per g of lignosulphonates.

9. A method according to any of claims 6-8, wherein the hydrogen peroxide source is hydrogen peroxide or a hydrogen peroxide precursor, e.g. perborate or percarbonate, or a

hydrogen peroxide generating enzyme system, e.g. an oxidase and its substrate, or a peroxycarboxylic acid or a salt thereof.

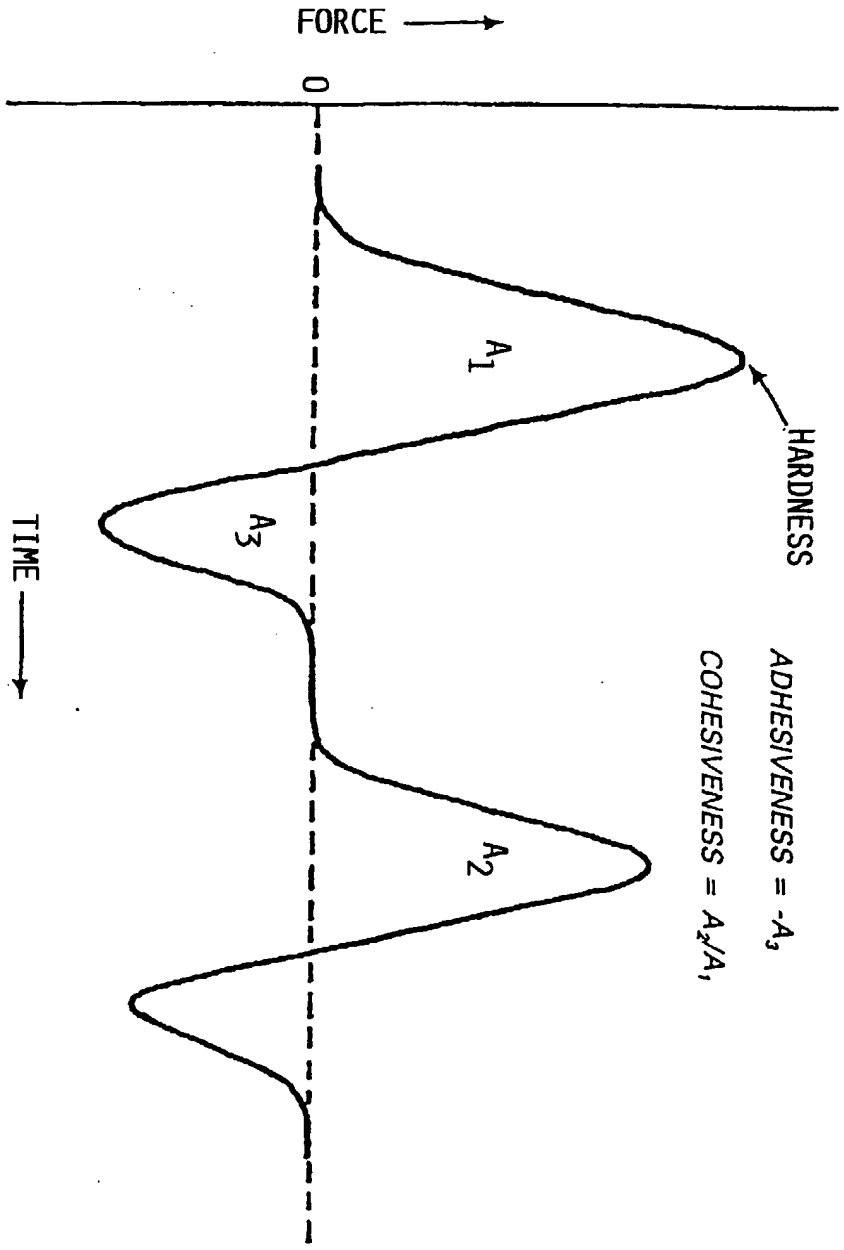
10. A method according to any of claims 6-9, in which the hydrogen peroxide source is added to the solution of lignosulphonates in a total amount corresponding to 0.05-5 mmoles per g of lignosulphonates.

11. A method according to any of claims 1-10, which additionally comprises an oxidizable substrate such as an organic compound, such as a phenolic compound, e.g. p-hydroxy-benzene sulphonate.

12. A method according to claim 11, wherein the amount of oxidizable substrate corresponds to a concentration in the solution of lignosulphonates of between 0.01 μM and 100 μM.

13. A gel preparation producible by the method according to any of claims 1-12.

1/2


FORCE
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COHESIVENESS = A2/A1

Fig. 1

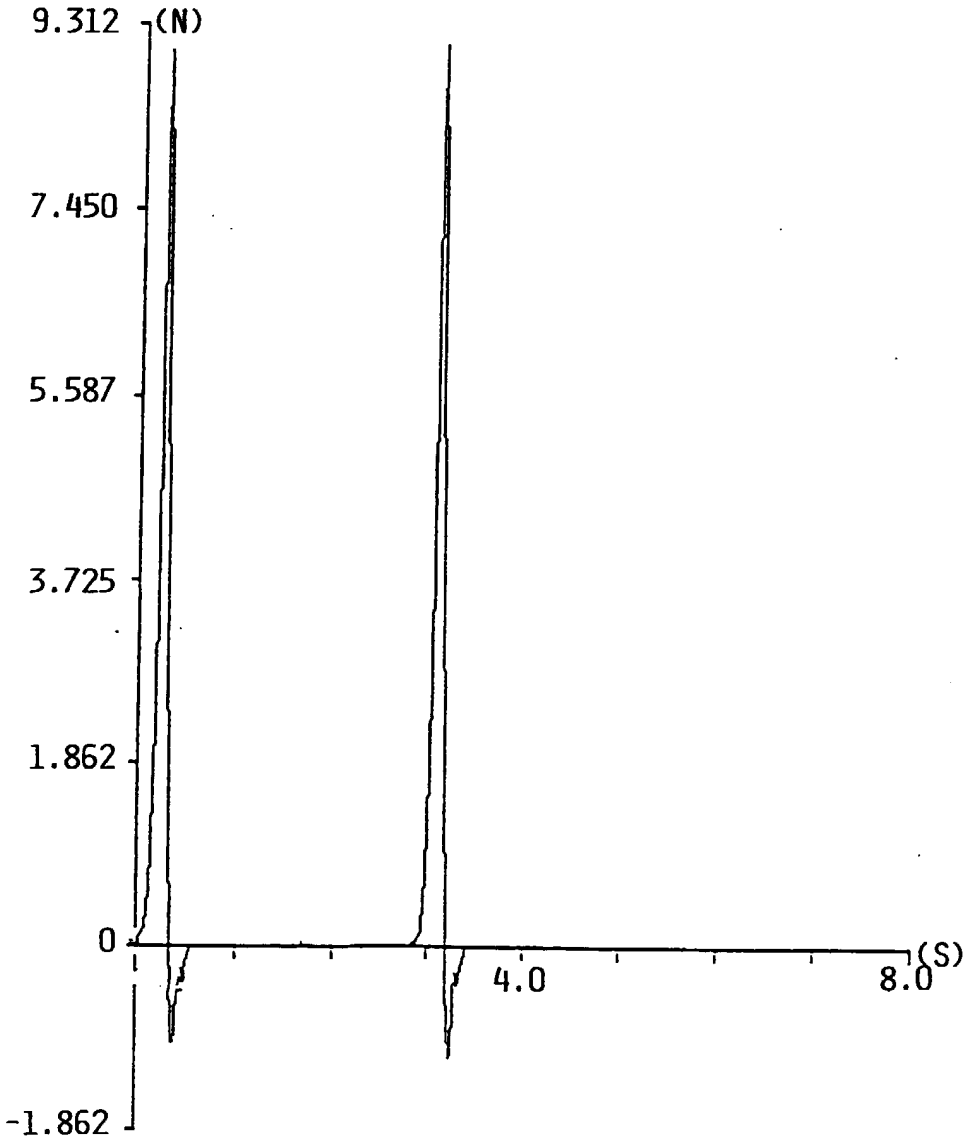

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00087

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12P 11/00, C12N 9/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12P, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, IFIPAT, CA, PAPERCHEM

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PHYTOCHEMISTRY, Volume 24, No 3, 1985, Andrzej Leonowicz et al, "The effect of fungal laccase on fractionated lignosulphonates (peritan Na)" page 393 - page 396 --	1-13
X	HOLZFORSCHUNG, Volume 36, 1982, Jerzy Lobarzewski et al, "The effects of fungal peroxidase on Na-lignosulfonates" page 173 - page 176 --	1-13
X	US, A, 4432921 (ANNEGRET HAARS ET AL), 21 February 1984 (21.02.84) --	1-13

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 June 1995	14 -06- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/DK 95/00087

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4432921	21/02/84	DE-A,C,C 3037992	19/08/82